Directed Evolution of Enzymes

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Critical Review

Directed Evolution of Enzymes: Library Screening Strategies

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Summary

Directed evolution has become the preferred engineering approach to generate tailor-made enzymes. The method follows the design guidelines of nature: Darwinian selection of genetic variants. This review discusses the different stages of directed evolution experiments with the focus on developments in screening and selection procedures. © 2009 IUBMB
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Keywords cell surface display; DNA shuffling; enzymology; error-prone PCR; high-throughput; *in vitro* compartmentalization; protein engineering; screening; saturation mutagenesis.

INTRODUCTION

Enzymes are nature's biocatalysts catalyzing chemical reactions at high velocity, with great specificity, under mild temperatures, with water as solvent. These reaction conditions are regarded as energy efficient and environmentally friendly. The number of commercial enzyme applications is continuously growing, despite the suboptimal performance of many natural enzymes under industrial process conditions. The current limitations of applications of enzymes in industry are poor stability, low reaction rates, product inhibition, and limited substrate conversion (*1*). In addition, there are many reactions for which no enzymes are known today. Protein engineers are therefore focusing on the identification of enzymes with new reaction specificities and are improving the performance of existing enzymes. Also, DNA databases and environmental DNA libraries are screened for better performing enzymes, even though newly identified enzymes are likely to require additional optimization via protein engineering. Since the 1980s, site-directed mutagenesis (rational design) has been used to improve the properties of enzymes. Often, this approach has met with limited success, mainly because of a general lack of understanding of how protein structure relates to enzyme function. Nature, in contrast, applies Darwinian selection, for example, survival of the fittest, to alter the properties of enzymes. Since the 1990s, the Darwinian selection strategy has been applied in laboratory evolution of proteins. This approach, called directed evolution, has quickly proven to be much more effective in enzyme engineering than rational design. Directed evolution involves the generation of random genetic diversity followed by high-throughput screening for desirable variants (Fig. 1) and requires no structural knowledge of the protein. Where structural information is available, rational design and directed evolution are often combined to create "smart libraries," introducing genetic variations at functional sites, such as the active site region of an enzyme.

LIBRARY CONSTRUCTION

The initial selection of parent gene and directed evolution method requires careful consideration by protein engineers, as these factors strongly influence the success of creating desirable enzyme variants. Parent enzymes should preferably possess the desirable activity at a low rate already, for example, as a promiscuous activity (*2*, *3*), or catalyze the desired chemistry on a related substrate. If two parents with similar initial catalytic properties are available, it is best to select the most stable one. Selection of a thermostable parent increases the chances for success in creating a desirable biocatalyst, as demonstrated for the cytochrome P450 BM3 enzyme (*4*): clearly, thermostable enzymes have a greater capacity to accept (structurally destabilizing) mutations over their mesophilic counterparts, and thus allow for a wider sampling of the mutational sequence space for catalytically favorable mutations.

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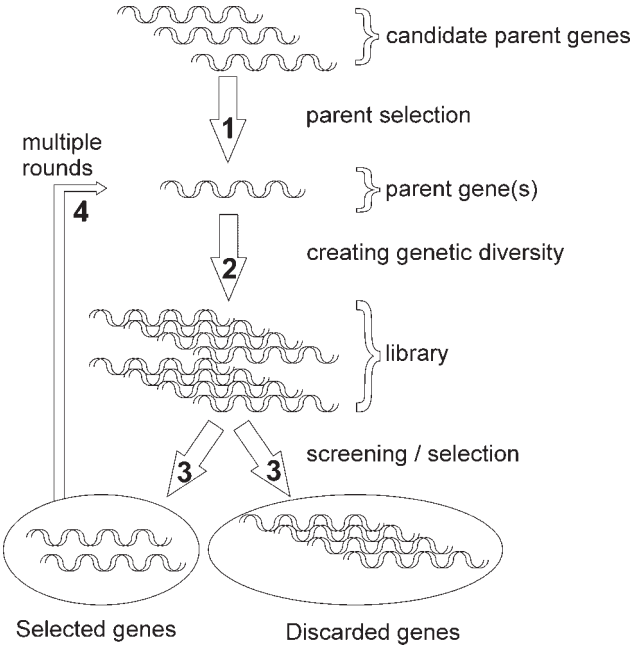

Figure 1. Flowchart of directed evolution.

In nature, genetic variation in DNA arises from errors introduced during genome duplication, or via DNA damaging by UV light or reactive chemicals. In the laboratory, however, random genetic variation in DNA nowadays is created by polymerase chain reaction (PCR) methods. The most common way of creating genetic variation is by *error-prone PCR*. This technique involves amplifying the gene in the presence of manganese ions or nucleotide analogs to promote the misincorporation of nucleotides by the DNA polymerase, or by using a low fidelity DNA polymerase such as MutazymeII (*5*).

Saturation mutagenesis is another library creation technique, which involves the randomization of one codon of a gene using synthetic oligonucleotides possessing a randomized codon flanked by wild-type sequences. Several codons may also be randomized, simultaneously, though this results in large libraries. Protein engineers may limit library size by reducing the degeneracy at targeted positions, for example by choosing a "NDT" codon (N: any nucleotide, D: adenine/guanine/thymine, and T: thymine) (*6*). The 12 possible codons encode 12 different amino acids. In contrast to error-prone PCR, saturation mutagenesis requires knowledge of functional important regions of the enzyme. Although studies have reported that saturation mutagenesis yielded better variants than error-prone PCR (*7–9*), this method relies heavily on structural insights for site specific targeting in proteins.

DNA-shuffling is also widely applied in the generation of mutant libraries and is based on homologous recombination of genes with high DNA sequence identity. Originally, this technique was used to randomly recombine various mutants of a single gene (*10*), for example, single gene shuffling. Later, DNA-shuffling was also shown to effectively recombine homologous genes of different microbial strains and species (*11*), for example, family shuffling. In a comparative study, variants obtained by family shuffling were shown to outperform variants obtained with single gene shuffling (*11*), though it requires the availability of multiple parents with high DNA sequence similarity. DNA-shuffling involves the controlled fragmentation of parent DNA, usually by DNAaseI. The resulting fragments of ~50 to 150 base pairs are then purified and serve as both PCR template and primers in a PCR gene assembly. In this way, advantageous mutations are effectively combined, while at the same time purging deleterious mutations, which is not possible with nonrecombinative methods such as error-prone PCR. The scope of DNA-shuffling can be extended by incorporating synthetic oligonucleotides carrying a randomized codon flanked by wild-type sequences in the gene assembly step. This allows the targeting of tens of codons in a single gene and gives control over the frequency at which targeted codons are mutated by varying the concentration of oligonucleotides added to the gene assembly PCR (*12*). Several other library creation techniques have been developed, such as circular permutation (*13*), random insertion and deletion of nucleotides (*14*), and random hybrid enzyme construction (*15*), but these techniques are rarely applied. The alternative to directed evolution is computational design of enzymes, though such designs typically show very low rate accelerations (*16*).

FINDING DESIRABLE ENZYMES

One of the most challenging steps of the directed evolution process is the identification of desirable variants from mutant libraries (*17*, *18*). An important consideration for protein engineers is whether or not to use model substrates possessing a chromophore or fluorophore for screening. Although these substrates are easier to work with, their use may optimize the enzyme for the model substrate and not for the real substrate. Various technologies are available to isolate variants of interest, which can be divided into "selection" versus "screening" and "*in vivo*" versus "*in vitro*" techniques (Table 1). The key elements of these approaches are discussed below.

SELECTIONS

Selection techniques rely on a direct link between cell growth and improved or acquired enzyme function (Fig. 2). Cells are transformed with the library followed by plating on selective medium. Selections are therefore limited to engineering detoxifying enzymes (*e.g.* β-lactamase (*10*)) or enzymes that synthesize essential nutrients for cell growth and survival. Recently, the enantioselectivity of a lipase was improved through the use of an aspartate auxotrophic *E. coli* strain. Cell growth was dependent on the hydrolysis of an enantiomeric pure aspartate ester by desirable lipase variants (*19*). As this selection strategy also selected for nonenantioselective lipases, a

Table 1
Comparison of screening and selection technologies

Strategy	Library size	Advantage	Disadvantage
Selection	$\sim 10^9$	Yields desirable variants only	Only possible if activity gives growth advantage
Agar plate screen	$\sim 10^5$	Simple to operate	Limited dynamic range
Microtiter plate screen	$\sim 10^4$	All analytical methods possible Excellent dynamic range	Relatively low screening capacity
Cell-in-droplet screen	$\sim 10^9$	Large libraries	Fluorescence detection and DNA modifying enzymes only
Cell as microreactor	$\sim 10^9$	Large libraries	Fluorescence detection only
Cell surface display	$\sim 10^9$	Large libraries	Fluorescence detection only
In vitro compartmentalization	$\sim 10^{10}$	No cloning steps Large libraries	Fluorescence detection and DNA modifying enzymes only

suicide inhibitor of the opposite enantiomer was included to inactivate lipases with activity toward the "incorrect" enantiomer, thus, inhibiting cell growth. Recently a similar selection strategy has been reported where lipase catalyzed hydrolysis of the "wrong" enatiomer yields a posion (fluoroacetic acid), whereas hydrolysis of the desirable enatiomer yields a carbon source (acetate) (*20*). Thus only cells expressing a lipase of desirable enatioselectivity can grow. Selection techniques are compatible with large libraries and do not require special instruments.

AGAR PLATE SCREENING

Agar plate screening is the most simplistic format of screening and involves the incubation of colonies with the enzyme substrate. The crucial factor with this screening technique is that substrate conversion creates a visual signal, such as fluorescence or color, to identify colonies expressing an enzyme with desirable properties (Fig. 2). Parikh and Matsumura (*21*) applied agar plate screening for the conversion of a β-galactosidase into a β-fucosidase. *E. coli* transformants ($\sim$1,000 per plate) were absorbed to filter paper and then incubated with 5-bromo-4-chloro-3-indoyl-β-D-fucopyranoside resulting in color development by colonies expressing β-fucosidase activity. A screening approach for glycosynthases was recently described which detects the release of hydrofluoric acid, the by-product of the glycosynthase reaction, via the pH indicator methyl red (*22*). *E. coli* colonies ($\sim$600) were pressed onto filter paper, lysed with liquid nitrogen, and then soaked in a substrate solution with methyl red. Colonies that turned red the quickest were selected, yielding a variant with 35-fold higher k_{cat} Van Loo et al. devised a screening system whereby epoxide hydrolase activity could be visualized on agar plates. Agar plates were incubated with epoxybutane vapor which was converted to the corresponding diol by functional epoxide hydrolase variants (*23*). *E. coli* oxidized the diol forming excess NADH which led to safranin O uptake, coloring colonies expressing active epoxide hydrolases. In general, agar plate screenings are easy to operate and are excellent in identifying active variants, but are weaker in visualizing differences in catalytic rates of enzyme variants.

MICROTITER PLATE SCREENING

Microtiter plate based screenings are the most commonly applied method amongst scientists in identifying desirable enzyme variants. Single transformants are grown in microtiter plates and variants are usually assessed in a second plate, after cell lysis, the original plate is stored as back-up (Fig. 2). A microtiter plate based screen was applied in the selection of cytochrome P450 BM3 variants capable of hydroxylating naproxen (*4*). *E. coli* transformants were grown in 96-well plates. Cells were pelleted and lysed followed by centrifugation. The remaining lysate was added to the substrate in a fresh plate. The hydroxylated naproxen was subsequently detected by reacting it with aminoantipyrene and reading the absorbance in a plate reader. Cyclodextrin glucanotransferase variants insensitive to the inhibitor acarbose and with 10-fold lower competing hydrolysis reaction were also selected through a microtiter-based screen (*24*, *25*). Error-prone PCR variants were expressed in *E. coli* grown in 96-well plates followed by lysis of cells before addition to the substrate starch. Reaction progress was monitored using a colorimetric assay for the formation of β-cyclodextrin and hydrolytic products. Williams et al. applied a 96-well plate screen to expand the substrate repertoire of a glycosyltransferase (*26*). Variants were expressed in *E. coli*, and lysates were used to test twenty different UDP-sugars as donor and 4-methylumbeliferone as acceptor. The 4-methylumbelliferone acceptor became nonfluorescent upon glycosylation, as detected by a fluorescence plate reader. Product formation in microtiter plates can also be visualized using antibodies, for example, ELISA (enzyme-linked-immunosorbent-assay). An error-prone PCR library of histone acetyltransferase was screened for thermostable variants, by growing *E. coli* expressing variants in 96-well plates followed by an ELISA (*27*). Cells

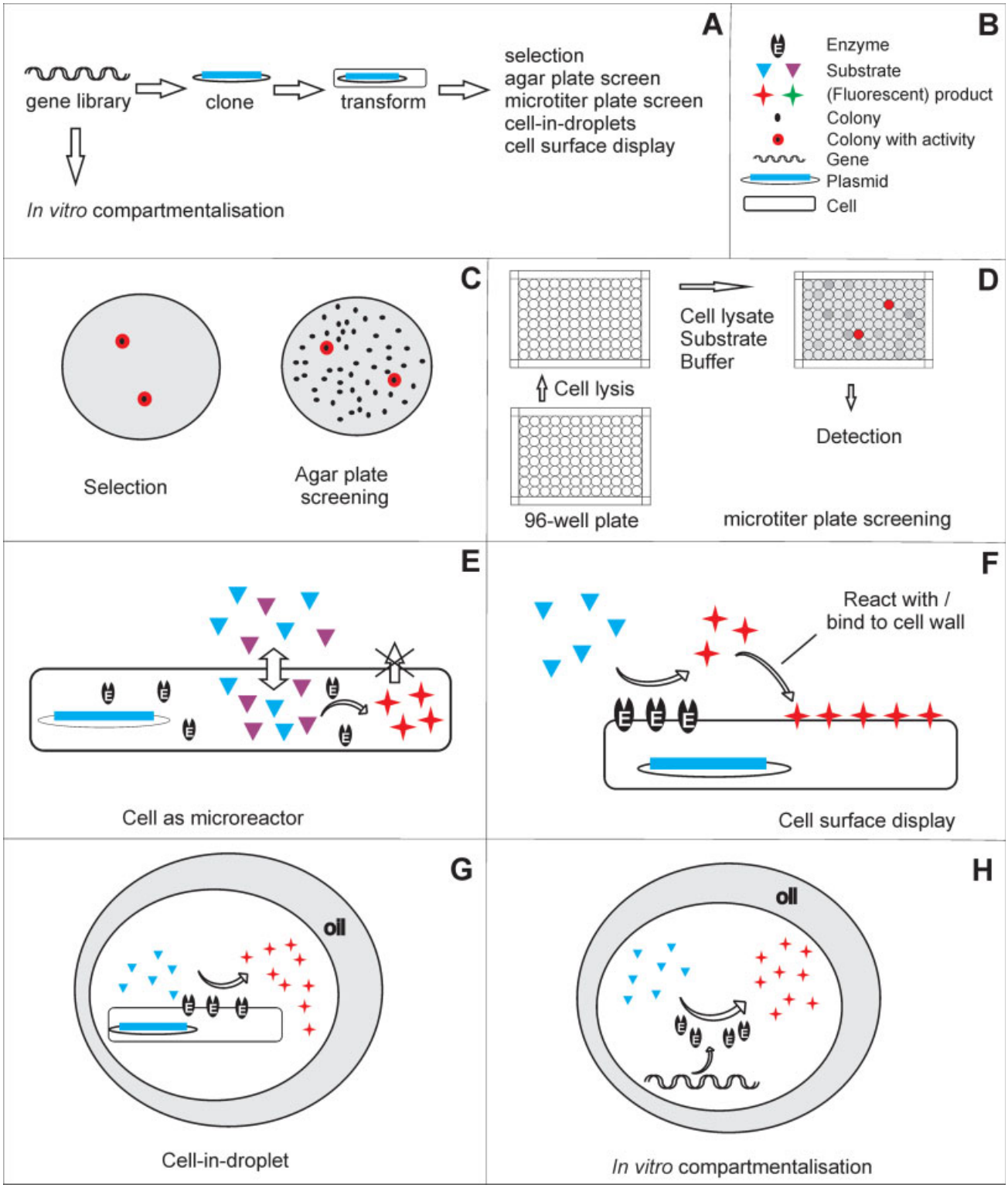

Figure 2. Overview of screening technologies. (A) Experimental steps from obtaining the gene library as PCR product to the actual screen. Note that cloning and transformation efficiency are often limiting library size, (B) explanation of symbols, (C) Cell growth/ survival selection and agar plate screening, (D) microtiter plate screening, (E) cell as micro-reactor, (F) cell surface display, (G) cell-in-droplet, and (H) *in vitro* compartmentalization. The methods are explained in the main text.

were pelleted and lysates added to microtiter plates coated with histone peptide substrates. The degree of histone acetylation was quantified by antibodies coupled to a peroxidase to allow for signal amplification using a chromogenic substrate. The contents of microtiter plate wells can also be analyzed by liquid/gas chromatography or mass spectrometry. Microtiter plate screenings thus enable the use of various analytical tools and whilst they offer a great dynamic range, their screening capacity is usually limited to less than 10^4 variants per day.

PICO AND FEMTO-LITER REACTORS: CELLS IN DROPLETS

Microtiter plate screening capacity can be increased by using smaller wells to increase their number on a plate; the company Diversa holds the record with a 1,000,000-well plate (*28*). Alternatively, the incorporation of single bacterial cells into droplets enables the formation of much smaller micro-reactors than microtiter plates allow. These droplets are water-in-oil compartments which hold together both cells, enzymes, substrates and products. Thermostable droplets are used to create numerous separate reaction vessels within a single PCR tube. This technique was applied to evolve DNA polymerase, via DNA shuffling, capable of bypassing lesions typical for ancient DNA (*29*). *E. coli* cells, each expressing a different DNA polymerase variant, were encapsulated in droplets of few pico-liter (most droplets contained a single *E. coli* cell) together with all PCR ingredients required to amplify the *Taq*-gene followed by 20 cycles of PCR. This screening method ensured that only *Taq*-genes encoding a functional DNA polymerase were amplified and thus enriched.

Single *E. coli* cells expressing serum paraoxonase variants were also encapsulated in droplets (water-in-oil) of ~10 femto-liter together with a thiolactonase substrate. Only droplets containing the desirable biocatalyst released a fluorophore, which remained "linked" to the cells via the droplet compartment (Fig. 2). The droplets were then emulsified forming water-in-oil-in-water droplets, followed by screening using a fluorescence-activated cell sorting system (FACS). Selected enzyme variants from a library population of $\sim 10^7$ displayed a 100-fold higher thiolactonase activity (*30*). This screening approach offers ultra-high-throughput, but requires fluorogenic substrates that do not cross the oil-phase barrier of the droplets.

Advances in microfluidic devices may overcome the current limitations of this technique. Huebner et al. (*31*) recently demonstrated that alkaline phosphatase activity expressed from a single *E. coli* cell encapsulated in a ~800 pico-liter droplet can be followed in time. Droplets were generated in microfluidic devices and the formation of a fluorescent reaction product within single droplets was detected via a microscope with a photomultiplier tube. This setup could easily discriminate between wild-type and a less active mutant. Coupled with a sorting device the technique creates a new screening platform for directed evolution (*31*). Moreover, the microfluidics platform gives exact control over the "micro-reactors" life-time, and allows for the addition of substrates and quenchers at desired time thus extending the scope of directed evolution screening strategies.

CELLS AS MICRO-REACTORS

Single bacterial cells may be regarded as the ultimate "micro-reactors" with volumes of few femto-liters only. The expression of a desirable biocatalyst should results in a "labeled" cell. Such a screen has been developed to select glycosyltransferase variants from an error-prone PCR library ($\sim 10^7$ members) that efficiently sialylated fluorescently labeled acceptor sugars (*32*). The critical part of this experiment is that both substrates are freely transported into and out of *E. coli*, whereas the fluorescent reaction product cannot, and thus accumulates in cells (Fig. 2) expressing the desirable variant, enabling selection by FACS.

CELL SURFACE DISPLAY

An alternative to the "micro-reactors" is cell surface display were both the enzyme variants and the substrates/products are displayed on the surface of cells (Fig. 2). By applying cell surface display, highly specific endopeptidase variants were selected for nonnative cleavage sites (*33*). Screening of variants was based on the cleavage of a "selection" peptide, indicating novel cleavage specificity, resulting in green fluorescence. At the same time wild-type specificity was counter selected via a second peptide that gave rise to a red fluorescence product. Both processed peptides carrying the fluorophores were positively charged and were thus captured on the surface of the negatively charged *E. coli*. The "green" *E. coli* cells expressing the desirable endopeptidase variants were isolated by FACS.

The enantioselectivity of horseradish peroxidase for L- over D-tyranisol was also improved, 8-fold, using cell surface display (*8*). Yeast cells displaying functional peroxidases converted fluorescently labeled L-tyranosil into a radical that reacted with tyrosine residues of cell surface proteins, thereby "coloring" the cells. In addition the amount of peroxidase displayed on individual cells was also visualized via a fluorescently labeled antibody directed against the C-myc epitope of the protein. Cells displaying the desirable biocatalyst were selected by FACS.

Recently, the enantioselectivity of an esterase was improved by displaying enzyme variants on the surface of *E. coli* and incubating the cells with equal amounts of differentially labeled ("red" and "green") S- and R-enantiomeric substrates (*34*). Esterase activity released a phenolic compound that was immediately attached to the cell surface via peroxidase catalyzed radical formation. FACS screening of $\sim 10^7$ variants yielded a mutant with an E_R of 15, whereas the wild-type showed no preference (E_R of 1). Cell surface display screening has a very high capacity (10^7 h^{-1}), but is limited by the requirement of fluorogenic substrates/products that stick to the cell surface.

IN VITRO SELECTION AND SCREENING

Apart from the cell based screening methods described above, a cell-free technology has been developed for ultra-high-throughput ($>10^7$ day^{-1}) screening for enzyme activities, called *in vitro* compartmentalization (*35*). Gene libraries (simply linear PCR products) along with an appropriate transcription/ translation mixture, and oil are emulsified yielding water-in-oil droplets of a few femto-liter. The genes are transcribed and translated into multiple copies of the encoded protein within the droplet compartments, thus ensuring genotype-phenotype linkage (Fig. 2). Note the similarity between this *in vitro* screening technique and the cell-in-droplet method (Fig. 2). Initially, *in vitro* compartmentalization had been designed to select DNA methyltransferases (*35*). Only genes encoding a desirable methyltransferase were methylated and thus survived incubation with a restriction enzyme specific for nonmethylated DNA after extraction of the DNA from the droplets. Later, *in vitro* compartmentalization was used to increase the promiscuous β-galactosidase activity of a protein with unknown function 4,000-fold (*36*). An error-prone PCR library was transcribed and translated in water-in-oil droplets together with fluorescein-β-D-galactopyranoside. Then water-in-oil-in-water droplets were prepared before the fluorescent droplets (carrying genes encoding β-galactosidase activity) were selected by FACS. To minimize leakage of the fluorophore from the droplets the oil-surfactant composition was optimized (*36*). More examples and details of *in vitro* compartmentalization technology, reporting on ribozyme, phosphotriesterase and restriction endonuclease selections and screenings, have been reviewed elsewhere (*37*, *38*). *In vitro* compartmentalization combines a very high screening capacity with the simplicity of library preparation (linear PCR products), whereas all cell based screening requires the cloning of genes into expression vectors.

FUTURE PROSPECTS

Nowadays, directed evolution is the method of choice for enzyme engineering. Over the last few years, several selection and screening methods have been reported allowing screening of over 10^7 variants per day. The more traditional microtiter plate screenings are medium-throughput, but are compatible with most analytical tools. In an ever-evolving climate of laboratory enzyme evolution, it is difficult to predict which of the screening technologies will dominate in the future. However, to expand the scope of ultra-high-throughput screenings, these methods should move outside the limitations of fluorescence detection only. A promising development in this field is the recent report on alkaline phosphatase expressed from single cells in droplets using microfluidics, as this technology has the potential to integrate alternative analytical tools. For example, coupling of these screening technologies to mass spectrometry would dramatically widen the scope of enzyme reaction products that can be detected. The development of such analytical tools will be valuable to advance synthetic biology.

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